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### Evidence for specific subunit distribution and interactions in the quaternary structure of $\alpha$ -crystallin

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### Abstract

The quaternary structure of  $\alpha$ -crystallin is dynamic, a property which has thwarted crystallographic efforts towards structural characterization. In this study, we have used collision-induced dissociation mass spectrometry to examine the architecture of the polydisperse assemblies of  $\alpha$ -crystallin. For total  $\alpha$ -crystallin isolated directly from fetal calf lens using size-based chromatography, the  $\alpha$ B-crystallin subunit was found to be preferentially dissociated from the oligomers, despite being significantly less abundant overall than the  $\alpha$ A-crystallin subunits. Furthermore, upon mixing molar equivalents of purified  $\alpha$ A- and  $\alpha$ B-crystallin, the levels of their dissociation were found to decrease and increase, respectively, with time. Interestingly though, dissociation of subunits from the  $\alpha$ A- and  $\alpha$ B-crystallin homo-oligomers was comparable, indicating that strength of the  $\alpha$ A: $\alpha$ A, and  $\alpha$ B: $\alpha$ B subunit interactions are similar. Taken together, these data suggest that differences in the number of subunit contacts in the mixed assemblies give rise to the disproportionate dissociation of  $\alpha$ B-crystallin subunits. Limited proteolysis mass spectrometry was also used to examine changes in protease accessibility during subunit exchange. The C-terminus of  $\alpha$ A-crystallin was more susceptible to proteolytic attack in homo-oligomers than that of  $\alpha$ B-crystallin. As subunit exchange proceeded, proteolysis of the  $\alpha$ A-crystallin C-terminus increased, indicating that in the hetero-oligomeric form this tertiary motif is more exposed to solvent. These data were used to propose a refined arrangement for the interactions of the  $\alpha$ -crystallin domains and C-terminal extensions of subunits within the  $\alpha$ -crystallin assembly. In particular we propose that the palindromic IPI motif of  $\alpha$ B-crystallin gives rise to two orientations of the C-terminus.

### Keywords

lens, alpha-crystallin, small heat shock protein, chaperone, mass spectrometry, quaternary structure

### Disciplines

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# **Evidence for Specific Subunit Distribution and Interactions in the Quaternary Structure of $\alpha$ -Crystallin**

Short Title: Subunit Interactions of  $\alpha$ -Crystallin

Key words: lens; alpha-crystallin; small heat shock protein; chaperone; mass spectrometry; quaternary structure.

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## Abstract

The quaternary structure of  $\alpha$ -crystallin is dynamic, a property which has thwarted crystallographic efforts towards structural characterization. In this study, we have used collision-induced dissociation mass spectrometry to examine the architecture of the polydisperse assemblies of  $\alpha$ -crystallin. For total  $\alpha$ -crystallin isolated directly from fetal calf lens using size-based chromatography, the  $\alpha$ B-crystallin subunit was found to be preferentially dissociated from the oligomers, despite being significantly less abundant overall than the  $\alpha$ A-crystallin subunits. Furthermore, upon mixing molar equivalents of purified  $\alpha$ A- and  $\alpha$ B-crystallin, the levels of their dissociation were found to decrease and increase, respectively, with time. Interestingly though, dissociation of subunits from the  $\alpha$ A- and  $\alpha$ B-crystallin homo-oligomers was comparable, indicating that strength of the  $\alpha$ A: $\alpha$ A, and  $\alpha$ B: $\alpha$ B subunit interactions are similar. Taken together, these data suggest that differences in the number of subunit contacts in the mixed assemblies give rise to the disproportionate dissociation of  $\alpha$ B-crystallin subunits. Limited proteolysis mass spectrometry was also used to examine changes in protease accessibility during subunit exchange. The C-terminus of  $\alpha$ A-crystallin was more susceptible to proteolytic attack in homo-oligomers than that of  $\alpha$ B-crystallin. As subunit exchange proceeded, proteolysis of the  $\alpha$ A-crystallin C-terminus increased, indicating that in the hetero-oligomeric form this tertiary motif is more exposed to solvent. These data were used to propose a refined arrangement for the interactions of the  $\alpha$ -crystallin domains and C-terminal extensions of subunits within the  $\alpha$ -crystallin assembly. In particular we propose that the palindromic IPI motif of  $\alpha$ B-crystallin gives rise to two orientations of the C-terminus.

## Introduction

Crystallins comprise approximately 90% of the total protein content of the vertebrate eye lens, and are highly concentrated in lens fibre cells, which typically contain no intracellular organelles<sup>1,2</sup>. Transparency of the lens is highly dependent on the packing, thus short-range order, of the crystallins<sup>3</sup>.  $\alpha$ -Crystallin makes up about one-third of the total crystallin content of the lens and exists as a multimer of homologous  $\alpha$ A- and  $\alpha$ B-crystallin subunits. In addition to the above structural role,  $\alpha$ -crystallin acts as a molecular chaperone by limiting the aggregation of unfolding proteins, thereby contributing to the extraordinary clarity of the healthy lens through decades of life. This functional role is particularly important as, unlike most organs of the body, little or no protein turnover occurs with age<sup>2</sup>.

On the basis of sequence homology and function,  $\alpha$ -crystallin has been classified as a member of the small heat shock protein (sHsp) family<sup>4</sup>, a group of molecular chaperones characterised by the presence of an evolutionarily conserved region of 80-100 amino acid residues, denoted the  $\alpha$ -crystallin domain<sup>5</sup>. sHsps derive their name from their small monomeric size, ranging from 12 kDa in *Caenorhabditis elegans* up to 43 kDa in *Saccharomyces cerevisiae*<sup>5</sup>, although they exist as oligomers of up to 50 subunits and 1.2 MDa in mass under physiological conditions<sup>6,7</sup>. The  $\alpha$ -crystallin domain is flanked by a relatively hydrophobic N-terminal domain and a highly polar C-terminal extension, both of which are variable in sequence and length<sup>8-10</sup>.

Contained within the variable C-terminal extension is an IXI/V motif, a conserved stretch of three hydrophobic amino acids that is essential for oligomerisation<sup>11</sup>. Modelling based on the crystal structures of Hsp16.5 and Hsp16.9 suggests that the IXI/V motif of one subunit interacts with a hydrophobic groove between  $\beta$ -sheet 4 and  $\beta$ -sheet 8, both in the  $\alpha$ -crystallin domain, of

another subunit<sup>12</sup>. Corresponding interactions have also been noted for  $\alpha$ A- and  $\alpha$ B-crystallin, suggesting that this interaction is not restricted to monodisperse, non-mammalian sHsps<sup>11</sup>.

Mammalian sHsps such as  $\alpha$ A- and  $\alpha$ B-crystallin and Hsp27 are highly dynamic and comprised of a variable number of subunits, properties which contribute to the polydisperse nature of the assemblies and thwart attempts at crystallisation. The quaternary structure of  $\alpha$ -crystallin is therefore a highly topical issue and many discrepant theoretical models have been proposed<sup>13-17</sup>.

We investigated the quaternary arrangement of  $\alpha$ -crystallin subunits by performing collision-induced dissociation mass spectrometry (CID-MS) on native  $\alpha$ -crystallin isolated from the fetal calf lens and during the subunit exchange reaction between purified  $\alpha$ A- and  $\alpha$ B-crystallin. In addition, we used limited proteolysis mass spectrometry (LP-MS) to examine solvent-exposed cleavage sites during subunit exchange between recombinant  $\alpha$ A- and  $\alpha$ B-crystallin. We found that there is a preferential dissociation of  $\alpha$ B-crystallin subunits during CID-MS of total  $\alpha$ -crystallin isolated from the lens. Furthermore, preferential truncation of the C-terminal extension of  $\alpha$ A-crystallin was observed as subunit exchange between  $\alpha$ A- and  $\alpha$ B-crystallin proceeded. From these data, we provide new insights into the subunit-subunit interactions within the quaternary structure of  $\alpha$ -crystallin.

## **Materials and Methods**

*Materials* – Expression vectors pET20b(+) and pET24d(+) (Novagen, San Diego, USA) containing the genes for bovine  $\alpha$ A-crystallin and human  $\alpha$ B-crystallin, respectively, were gifts from Joseph Horwitz (UCLA School of Medicine) and Teresa Treweek (University of Wollongong), respectively. Recombinant bovine  $\alpha$ B-crystallin and Hsp27 proteins were gifts

from Wilbert Boelens (Radboud Universiteit Nijmegen). Trypsin from bovine pancreas was purchased from Worthington Biochemical Corp. (Lakewood, USA). All reagents used were of analytical grade.

*Purification of  $\alpha$ -Crystallin Proteins* – Total  $\alpha$ -crystallin was isolated from fetal calf lenses as described previously<sup>18</sup>. Acid denaturation of the isolated  $\alpha$ -crystallin was performed as described previously<sup>19</sup>.  $\alpha$ A- and  $\alpha$ B-crystallin were purified from fetal calf lenses under denaturing conditions and subsequently refolded as described previously<sup>19</sup>. pET20b(+)- $\alpha$ A-crystallin (bovine) and pET24d(+)- $\alpha$ B-crystallin (human) DNA were transformed into electrically competent BL21(DE3) *Escherichia coli* before expression. Transformed cells were grown in Luria–Bertani medium containing 0.4% (w/v) glucose and antibiotics (100  $\mu$ g/mL ampicillin and 50  $\mu$ g/mL kanamycin, respectively). Protein expression was induced with 0.4 mM isopropyl thio- $\beta$ -D-galactoside. Purification of expressed  $\alpha$ A- and  $\alpha$ B-crystallin was performed according to the method described by Horwitz *et al.*<sup>20</sup>. Following the final chromatography step, fractions containing  $\alpha$ A- or  $\alpha$ B-crystallin were concentrated, exchanged into MilliQ water and lyophilized. The purity of recombinant proteins was confirmed by MS using nanoelectrospray ionisation (nanoESI-MS).

*Subunit Exchange of  $\alpha$ A- and  $\alpha$ B-Crystallin* – Subunit exchange experiments were performed using either proteins purified from fetal calf lens or recombinant proteins. Homogeneous solutions of  $\alpha$ A- and  $\alpha$ B-crystallin were prepared in 200 mM  $\text{NH}_4\text{OAc}$  (pH 7.0) and their concentrations determined from  $A_{280}$  values using the extinction co-efficient of 0.85 for a 1 mg/mL solution<sup>21</sup>. The solutions and buffer were equilibrated on ice for several minutes before being combined in a 1:1 (w/w) ratio with a total  $\alpha$ -crystallin concentration of 1.6 mg/mL and

1 mg/mL for CID-MS and LP-MS experiments, respectively. Subunit exchange was initiated by incubation of the mixtures at 37°C. Aliquots were taken at various time points and kept on ice to halt subunit exchange. Similarly, subunit exchange experiments between recombinant  $\alpha$ A- and  $\alpha$ B-crystallin and between Hsp27 and  $\alpha$ B-crystallin were performed at 43°C.

*Collision-Induced Dissociation Mass Spectrometry (CID-MS)* – CID-MS was performed on total  $\alpha$ -crystallin isolated from fetal calf lenses and quenched subunit exchange samples between lens-purified  $\alpha$ A- and  $\alpha$ B-crystallin at 37°C, recombinant  $\alpha$ A- and  $\alpha$ B-crystallin at 43°C and recombinant Hsp27 and  $\alpha$ B-crystallin at 43°C on modified Q-Tof 2 or Q-Tof Ultima™ mass spectrometers (Waters, Milford, USA) as described<sup>19</sup>. The following instrument parameters were used: capillary voltage 1.6 kV; sample cone 200 V; extractor cone 10 V; ion transfer stage pressure  $1.1 \times 10^{-3}$  mbar; quadrupole analyzer pressure  $8.7 \times 10^{-3}$  mbar and ToF analyzer pressure  $1.66 \times 10^{-6}$  mbar. To sequentially dissociate up to three monomers from the oligomers, the voltage applied to the collision cell was 200 V. Typically, 2  $\mu$ L of solution was electrosprayed from gold-coated glass capillaries prepared in-house.

*Limited Proteolysis Mass Spectrometry* – LP-MS was performed on cold-quenched subunit exchange reactions between recombinant  $\alpha$ A- and  $\alpha$ B-crystallin proteins at 37°C. Digestion was initiated by the addition of trypsin in a protease: $\alpha$ -crystallin ratio of 1:25 (w/w). Samples were digested at 10°C for 15 min. Digestion was quenched by the addition of 1% formic acid, 90% acetonitrile in a ratio of 1:1.25 (v/v) quenching solution:digest reaction. Two independent experiments were performed. Mass spectra of digested samples were acquired on a SYNAPT™ HDMS™ mass spectrometer (Waters) using a nanoESI source. The following instrument parameters were used: capillary voltage 1.5 kV; sample cone 40 V; extraction cone 4 V and



transfer collision energy 4 V. Typically, 2  $\mu$ L of solution was electrosprayed from gold-coated glass capillaries prepared in-house.

*Protein Quantification* – All mass spectra were externally calibrated with a solution of either sodium iodide or cesium iodide and processed using MassLynx™ software (Waters) with minimal smoothing and without background subtraction. The relative abundance of each protein species was determined by summing the intensities of eight of the most significant peaks of each protein envelope in the smoothed and centred spectrum. For the LP-MS samples, some peaks belonged to more than one protein envelope and it was not possible to determine the intensity of the constituent peaks, thus overlapping peaks were not included. To ensure consistency, the same peaks were summed for each spectrum. For acid-denatured  $\alpha$ -crystallin and CID experiments, the abundances of  $\alpha$ A- and  $\alpha$ B-crystallin were expressed as a proportion of total protein. For LP experiments, the abundances of truncated  $\alpha$ A- and  $\alpha$ B-crystallin proteins were expressed as a proportion of total  $\alpha$ A- and  $\alpha$ B-crystallin, respectively.

## **Results**

*Dissociation of Subunits from the Native  $\alpha$ -Crystallin Assembly* –  $\alpha$ -Crystallin in the mammalian lens is comprised of  $\alpha$ A- and  $\alpha$ B-crystallin subunits in a ratio of approximately 3:1<sup>22</sup>. Mass spectrometry of acid-denatured fetal calf lens  $\alpha$ -crystallin, in which the assembly was completely dissociated into monomers, showed the presence of four major protein species:  $\alpha$ A- and  $\alpha$ B-crystallin and singly phosphorylated forms of each subunit (Fig. 1A). Phosphorylation occurs early during lens development, with a proportion of phosphorylated  $\alpha$ -crystallin present in pre-natal lenses, as evidenced here<sup>23,24</sup>. It is clear from the transformed spectra (Fig. 1, insets),

that the ratios of phosphorylated to non-phosphorylated species were relatively consistent, indicating that the phosphorylated species did not ionize or dissociate in a grossly different manner. A C-terminally truncated form of  $\alpha$ A-crystallin,  $\alpha A_{1-151}$  was also identified in the spectrum. This truncation product was present in relatively low abundance and is likely a result of either in-source fragmentation and/or acid-induced cleavage associated with denaturation using hydrogen exchange resin.

Taking all protein species into account, the signal intensity arising from  $\alpha$ A-crystallin was significantly greater than that from  $\alpha$ B-crystallin (Fig. 1A, inset). Quantification of the respective ions revealed that 65% of the signal was attributable to  $\alpha$ A-crystallin, reflecting the previously reported abundance of this subunit in the mammalian lens<sup>25</sup>.

CID-MS of  $\alpha$ -crystallin and other multimeric proteins results in the sequential dissociation of subunits from the assemblies<sup>26</sup>. By manipulating the voltage applied to the collision cell, oligomers can be stripped of one, two or three monomers, depending upon the amount of internal energy generated via collisions with the noble gas ions in the collision cell. CID-MS of isolated fetal calf lens  $\alpha$ -crystallin performed under non-denaturing conditions resulted in 63% of the dissociated monomer signal arising from  $\alpha$ B-crystallin (Fig. 1B). This is compared with the 35% relative signal intensity observed for  $\alpha$ B-crystallin in the fully denatured protein mixture (Fig. 1A). Thus, although there is clearly a greater overall proportion of  $\alpha$ A-crystallin subunits in the fetal lens isolate, there is a significant preferential dissociative loss of  $\alpha$ B-crystallin subunits from the native assemblies during CID-MS.

*Dissociation of Subunits during Subunit Exchange* – In an attempt to understand the preferential dissociation of  $\alpha$ B-crystallin from the native  $\alpha$ -crystallin assembly, and to investigate changes in the quaternary arrangement of  $\alpha$ -crystallin during subunit exchange, we performed CID-MS at various time points after mixing solutions of  $\alpha$ A- and  $\alpha$ B-crystallin purified from fetal calf lens. Upon mixing,  $\alpha$ -crystallin oligomers undergo subunit exchange, the dynamics of which are highly temperature-dependent, with the rate of exchange increasing with temperature, and no observable subunit exchange occurring at 3°C<sup>27,28</sup>. We exploited this property in our experimental design such that subunit exchange could be delayed or stopped by controlling the temperature of the sample. The  $\alpha$ A- and  $\alpha$ B-crystallin homo-oligomers were combined in a ratio of 1:1 and subunit exchange was allowed to proceed at 37°C, with aliquots being quenched on ice at intervals. The relative signal intensities arising from  $\alpha$ A- and  $\alpha$ B-crystallin were determined from peak heights of the corresponding ions (Fig. 2). Prior to subunit exchange, the signal intensities of the  $\alpha$ A- and  $\alpha$ B-crystallin subunits were comparable (51% and 49%, respectively) (Fig. 2, inset). As subunit exchange proceeded, the abundance of dissociated  $\alpha$ B-crystallin subunits increased, with 86% of the signal arising from  $\alpha$ B-crystallin peaks after 60 min. At this time, a plateau was reached (not shown), indicating that, in agreement with Bova *et al.*<sup>27</sup>, subunit exchange was complete in approximately one hour. Similar trends were observed for subunit exchange reactions involving recombinant human  $\alpha$ B-crystallin homo-oligomers, with the  $\alpha$ B-crystallin signal intensity increasing by 35% and 20%, respectively, after complete exchange with recombinant bovine  $\alpha$ A-crystallin and human Hsp27 (not shown). The increase in dissociation of  $\alpha$ B-crystallin subunits during subunit exchange with  $\alpha$ A-crystallin and Hsp27 indicates that the preferential loss of  $\alpha$ B-crystallin subunits also occurs in partially-

exchanged oligomers, and that this property is not restricted to lens-isolated  $\alpha$ -crystallin, nor to assemblies with a 3:1 subunit ratio.

*Subunit Exchange and Limited Proteolysis* – To further investigate the changes in the quaternary structure of  $\alpha$ -crystallin as it undergoes subunit exchange, we performed LP-MS over the time course of the subunit exchange reaction. Subunit exchange between a 1:1 mixture of recombinant bovine  $\alpha$ A- and human  $\alpha$ B-crystallin was initiated and allowed to proceed at 37°C, with aliquots being quenched on ice at various time points. These samples were then subjected to limited proteolysis by incubation with trypsin at 10°C for 15 min. Based on the findings of Bova *et al.*<sup>27</sup>, subunit exchange at this temperature is negligible. Quenching of the digestion was achieved by acidification, which denatured both the trypsin and the  $\alpha$ -crystallin mixture.

MS analysis of the quenched digestions yielded peaks arising from full-length proteins, as well as the C-terminal truncation products  $\alpha$ A<sub>1-157</sub>,  $\alpha$ B<sub>1-157</sub> and  $\alpha$ B<sub>1-174</sub> (Fig. 3). LP-MS of the sample taken immediately after mixing the homo-oligomeric solutions (i.e. prior to subunit exchange), revealed that 55% of the total  $\alpha$ A-crystallin signal arose from the C-terminally truncated form of this subunit and 36% of the  $\alpha$ B-crystallin signal arose from the  $\alpha$ B-crystallin truncation products (Fig. 4). This suggests that, in the homo-oligomeric arrangements, the C-terminus of  $\alpha$ A-crystallin is more exposed to protease than that of  $\alpha$ B-crystallin. The relative abundance of truncated  $\alpha$ A-crystallin increased as the subunit exchange reaction proceeded, with the truncation accounting for 61% of the  $\alpha$ A-crystallin signal at 55 min, whilst the corresponding  $\alpha$ B-crystallin truncation decreased in abundance to 27% of the  $\alpha$ B-crystallin signal at 55 min. A plateau region occurred after this time (not shown). Thus, the C-terminus of  $\alpha$ A-crystallin is

somewhat more susceptible to proteolytic attack in the hetero-oligomeric form than in the homo-oligomer, with the opposite being the case for  $\alpha$ B-crystallin.

## Discussion

CID-MS of  $\alpha$ -crystallin results in the dissociation of one or more monomers from each oligomeric assembly, whilst acidification of  $\alpha$ -crystallin denatures the assemblies such that all of the constituent subunits are present in their unfolded monomeric forms. Random dissociation of subunits from the native  $\alpha$ -crystallin assemblies during CID-MS would be expected to result in ion distribution series in the monomeric region quantitatively resembling the overall total population of subunits, i.e. the signal intensities of the dissociated subunits would be in the same proportion as in the denatured sample. This was not the case, however, as a significantly greater abundance of  $\alpha$ B-crystallin monomers was present during CID-MS, indicative of a preferential dissociation of  $\alpha$ B-crystallin from the native assemblies. This phenomenon was also observed for total  $\alpha$ -crystallin formed after complete subunit exchange of purified recombinant homo-oligomers combined in a 1:1 ratio (Fig. 2).

It has been established that  $\alpha$ A- and  $\alpha$ B-crystallin homo-oligomers have a propensity to form assemblies with an even number of subunits and thus have a dimeric substructure, a property that is lost by  $\alpha$ B-crystallin upon phosphorylation<sup>29,30</sup>. Since the phosphorylation sites of  $\alpha$ B-crystallin (residues 19, 45 and 59) are all in the N-terminal domain, the dimeric preference is most likely a result of interactions between the N-terminal domains of adjacent subunits within the  $\alpha$ -crystallin oligomer. Indeed, stretches of residues between the phosphorylation sites at

residues 45 and 59 have been shown to be necessary for  $\alpha$ B-crystallin to interact with  $\alpha$ A-crystallin and undergo subunit exchange<sup>31-33</sup>.

Because of the high variability between the N-terminal domains of even closely related sHsps, the properties of inter-subunit interactions involving these domains are presumably dependent upon the subunits involved<sup>10</sup>. The preferential dissociation of  $\alpha$ B-crystallin subunits from fetal lens-isolated  $\alpha$ -crystallin, as well as hetero-oligomers formed by subunit exchange between both lens-purified and recombinant  $\alpha$ A- and  $\alpha$ B-crystallin homo-oligomers, demonstrates that there are non-equivalent associations between the  $\alpha$ A- and  $\alpha$ B-crystallin subunits. This is evidenced by the comparable dissociation of monomers from the respective homo-oligomers prior to mixing (Fig 2, inset). The fact that  $\alpha$ B-crystallin subunits dissociate more readily from the mixed oligomers may be explained by the  $\alpha$ A-crystallin subunits forming a core within the assembly, with  $\alpha$ B-crystallin subunits arranged in more exposed quaternary positions. Alternatively, it may suggest that the  $\alpha$ A: $\alpha$ B subunit interactions are weaker than  $\alpha$ A: $\alpha$ A and  $\alpha$ B: $\alpha$ B, and that on average  $\alpha$ B-crystallin subunits experience a greater proportion of weaker  $\alpha$ A: $\alpha$ B contacts than do the  $\alpha$ A-crystallin subunits.

The structure of mammalian sHsps is dynamic and the polydispersity of  $\alpha$ -crystallin is well-documented<sup>19,29,34</sup>. It is therefore probable that there is no single arrangement of subunits that make up the quaternary structure of  $\alpha$ -crystallin, however, cryo-EM data suggest that a 24mer is the predominant oligomer<sup>35</sup>. Based on our findings, we have proposed a possible arrangement of  $\alpha$ -crystallin subunits that allows for a variable number of subunits and  $\alpha$ A: $\alpha$ B ratios (Fig. 5). Subunits are arranged in a manner similar to that observed for Hsp16.5<sup>36</sup>. In this conformation, the  $\alpha$ A-crystallin subunits experience more inter-subunit contacts than the  $\alpha$ B-crystallin

subunits. Whilst all subunits have approximately the same number of weaker  $\alpha A:\alpha B$  interactions, the  $\alpha B$ -crystallin subunits have fewer homologous contacts than the  $\alpha A$ -crystallin subunits, thus are less tightly bound to the assembly.

Whilst CID-MS provided information on interactions between subunits in the  $\alpha$ -crystallin assembly, LP-MS experiments were undertaken in order to examine quaternary and tertiary accessibility of trypsin to the subunits as subunit exchange proceeded. After LP-MS, full-length  $\alpha A$ - and  $\alpha B$ -crystallin proteins were identified, as well as C-terminally truncated forms of each. Peaks corresponding to truncations in other regions of the subunits were not observed. This is consistent with the presence of a highly flexible, solvent-exposed region in the C-terminal extension of mammalian sHsps, which is more susceptible to proteolytic attack than other regions of the protein<sup>8,37</sup>.

Prior to subunit exchange, approximately half of the  $\alpha A$ -subunits in the homo-oligomers were present as the truncation product  $\alpha A_{1-157}$ . This was significantly greater than the relative abundance of truncated  $\alpha B$ -crystallin ( $\alpha B_{1-157}$  and  $\alpha B_{1174}$ ), indicating that R157 in the C-terminal extension of  $\alpha A$ -crystallin is more accessible to protease than this residue in  $\alpha B$ -crystallin homo-oligomers. Potential differences between the orientation of the C-terminal extensions of  $\alpha A$ - and  $\alpha B$ -crystallin are shown in Fig. 6. We propose that the IXI/V motif of  $\alpha A$ -crystallin (IPV) interacts with its binding site in a neighbouring subunit such that the flexible region of the C-terminal extension is oriented towards the interior of the oligomer (Fig. 6A). The IXI/V motif of  $\alpha B$ -crystallin (IPI) is a palindrome, and is thus likely to be able to interact with its binding site bi-directionally, as does the palindromic IXI/V motif of Hsp 16.9<sup>12,38</sup>. Thus, the C-terminal extension of  $\alpha B$ -crystallin can adopt the same orientation as in  $\alpha A$ -crystallin

(Fig. 6B) or with the IXI/V motif oriented in the opposite direction and the flexible region of the C-terminus extended towards the exterior (Fig. 6C).

In these configurations, the observed tryptic cleavage site at R157 is located on a loop that extends between the monomers, with the R157 residue more accessible in  $\alpha$ A-crystallin (Fig. 6A), as well as the  $\alpha$ B-crystallin subunit oriented similarly to  $\alpha$ A-crystallin (Fig. 6B). With a proportion of  $\alpha$ B-crystallin subunits having cleavage sites oriented towards the interior of the oligomer, and thus more shielded from the protease (Fig. 6C), the R157 residue of  $\alpha$ A-crystallin is overall more exposed than that of  $\alpha$ B-crystallin.

Several other potential tryptic cleavage sites exist in the C-terminal extensions of  $\alpha$ A- and  $\alpha$ B-crystallin: R163 and K166, and also K174 for  $\alpha$ B-crystallin. The relative abundance of  $\alpha$ B<sub>1-174</sub> did not change during subunit exchange, consistent with K174 being the penultimate residue of  $\alpha$ B-crystallin and thus highly flexible and accessible to solvent at all times<sup>39</sup>. Cleavage at R163 or K166 was not observed for either  $\alpha$ A- or  $\alpha$ B-crystallin. Disordered structure in the C-terminal extensions of  $\alpha$ A- and  $\alpha$ B-crystallin has been shown to commence at K166<sup>24</sup>. Because the preceding residue is less mobile and also adjacent to the IXI/V motif, which associates with the globular structure of the adjacent monomer, it is likely that K166 is inaccessible to trypsin as a result of steric hindrance. R163 is not a flexible residue and is also in close proximity to the interacting IXI/V motif and the globular  $\alpha$ -crystallin domains.

The proposed orientations of the C-terminal extensions allow the flexible region of  $\alpha$ B-crystallin to be more exposed in some of the subunits than that of  $\alpha$ A-crystallin. The greater overall accessibility of the flexible region of  $\alpha$ B-crystallin may be an important feature contributing to



previous observations that the extension of  $\alpha$ B-crystallin loses flexibility in the presence of target proteins and that the C-terminal lysine residue of  $\alpha$ B-crystallin may be involved in interactions, properties which  $\alpha$ A-crystallin lack<sup>40,41</sup>.

As subunit exchange proceeded, the proportion of truncated  $\alpha$ A-crystallin increased whilst that of  $\alpha$ B-crystallin decreased. This is indicative of the cleavage site of  $\alpha$ B-crystallin, already in a relatively protected environment in the homo-oligomer compared with that of  $\alpha$ A-crystallin, becoming more shielded during subunit exchange. The  $\alpha$ -crystallin hetero-oligomer is larger than the  $\alpha$ A- and  $\alpha$ B-crystallin homo-oligomers and is more tightly packed<sup>8,42</sup>. Thus, it is feasible that this tighter packing of the hetero-oligomer further shields the cleavage site of the  $\alpha$ B-crystallin extension from protease.

In the absence of direct visualisation of the quaternary structure of  $\alpha$ -crystallin, we have used MS methodologies to propose an arrangement of subunits within the  $\alpha$ -crystallin oligomer. In this representation, the  $\alpha$ B-crystallin subunits experience fewer contacts than the  $\alpha$ A-crystallin subunits, giving rise to the preferential dissociation of  $\alpha$ B-crystallin from the  $\alpha$ -crystallin complex. Preferential truncation of the C-terminal extension of  $\alpha$ A-crystallin is highly suggestive of different accessibilities of the R157 cleavage site between the extensions of  $\alpha$ A- and  $\alpha$ B-crystallin. Differential interactions between the IXI/V motifs of  $\alpha$ A- and  $\alpha$ B-crystallin are emphasised, but this warrants further investigation. To the authors' knowledge, only one study has used mutagenesis to investigate the role of the palindromic IPI motif of  $\alpha$ B-crystallin, however this work replaced one palindromic motif with another (GPG), thus not specifically addressing the palindromic role<sup>11</sup>. We plan to further investigate this by introducing single

mutations at residue 159 or 161 in  $\alpha$ B-crystallin and studying the dynamics of subunit exchange and oligomeric stability using the methodology of the present work.

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## Figure Legends

**Fig. 1:** Smoothed mass spectra of the monomeric region of native  $\alpha$ -crystallin isolated from fetal calf lens after acid denaturation (A) and dissociation by CID-MS (B). Only a portion of the monomeric region is shown for clarity. Some peaks arising from the proteins present are indicated:  $\alpha$ A-crystallin (open stars),  $\alpha$ B-crystallin (filled stars), phosphorylated  $\alpha$ A-crystallin (open circles), phosphorylated  $\alpha$ B-crystallin (filled circles) and  $\alpha$ A<sub>1-151</sub> (open squares). Transformed spectra are shown in inset.

**Fig. 2:** Representative smoothed mass spectra of a portion of the monomeric region of subunit exchange between lens-purified  $\alpha$ A- and  $\alpha$ B-crystallin after CID-MS. Only the major peaks are shown for clarity and some peaks arising from  $\alpha$ A- and  $\alpha$ B-crystallin are indicated. Spectra shown are from CID-MS of samples after 0 (back), 7.5, 15, 30 and 60 min (front) subunit exchange. *Inset:* Relative signal intensity arising from the dissociated monomeric  $\alpha$ A- (open circles) and  $\alpha$ B-crystallin (filled circles) during the process of subunit exchange compared with total protein signal.

**Fig. 3:** Representative smoothed mass spectrum of the monomeric region after 15 min of subunit exchange between recombinant bovine  $\alpha$ A- and human  $\alpha$ B-crystallin at 37°C after LP-MS. Peaks that were used to quantify protein species are indicated:  $\alpha$ A-crystallin (open stars),  $\alpha$ B-crystallin (filled stars),  $\alpha$ A<sub>1-157</sub> (open circles) and  $\alpha$ B<sub>1-157</sub> (filled circles).

**Fig. 4:** Relative signal intensity of C-terminal truncations of  $\alpha$ A- (open circles) and  $\alpha$ B-crystallin (filled circles) present after LP-MS as a function of subunit exchange time. Proportions are the

amount of truncated proteins in relation to total  $\alpha$ A- or  $\alpha$ B-crystallin. Data are the means of duplicate experiments  $\pm$  SEM.

Fig. 5: A possible arrangement of the subunits within the  $\alpha$ -crystallin oligomer highlighting differences in interactions between  $\alpha$ A- (purple) and  $\alpha$ B-crystallin (blue) subunits. The  $\alpha$ A-crystallin subunits form the core of the structure, with the  $\alpha$ B-crystallin subunits in more exposed locations, with fewer inter-subunit contacts. The N-terminal domains (darker spheres) are oriented towards the interior of the oligomers and are surrounded by the C-terminal domains (lighter spheres). C-terminal extensions are not shown. Adapted from the octahedral symmetry arrangement for Hsp16.5<sup>36</sup>.

Fig. 6: Differences between the orientation of the C-terminal extensions of  $\alpha$ A- (A) and  $\alpha$ B-crystallin (B and C) highlighting interactions between the IXI/V motif of one oligomer and the IXI/V binding site (black) on the adjacent oligomer, and the resultant orientation of the C-terminal extension (dotted line represents the flexible region that protrudes from the oligomer). The R157 trypsin cleavage site is denoted by a red X.

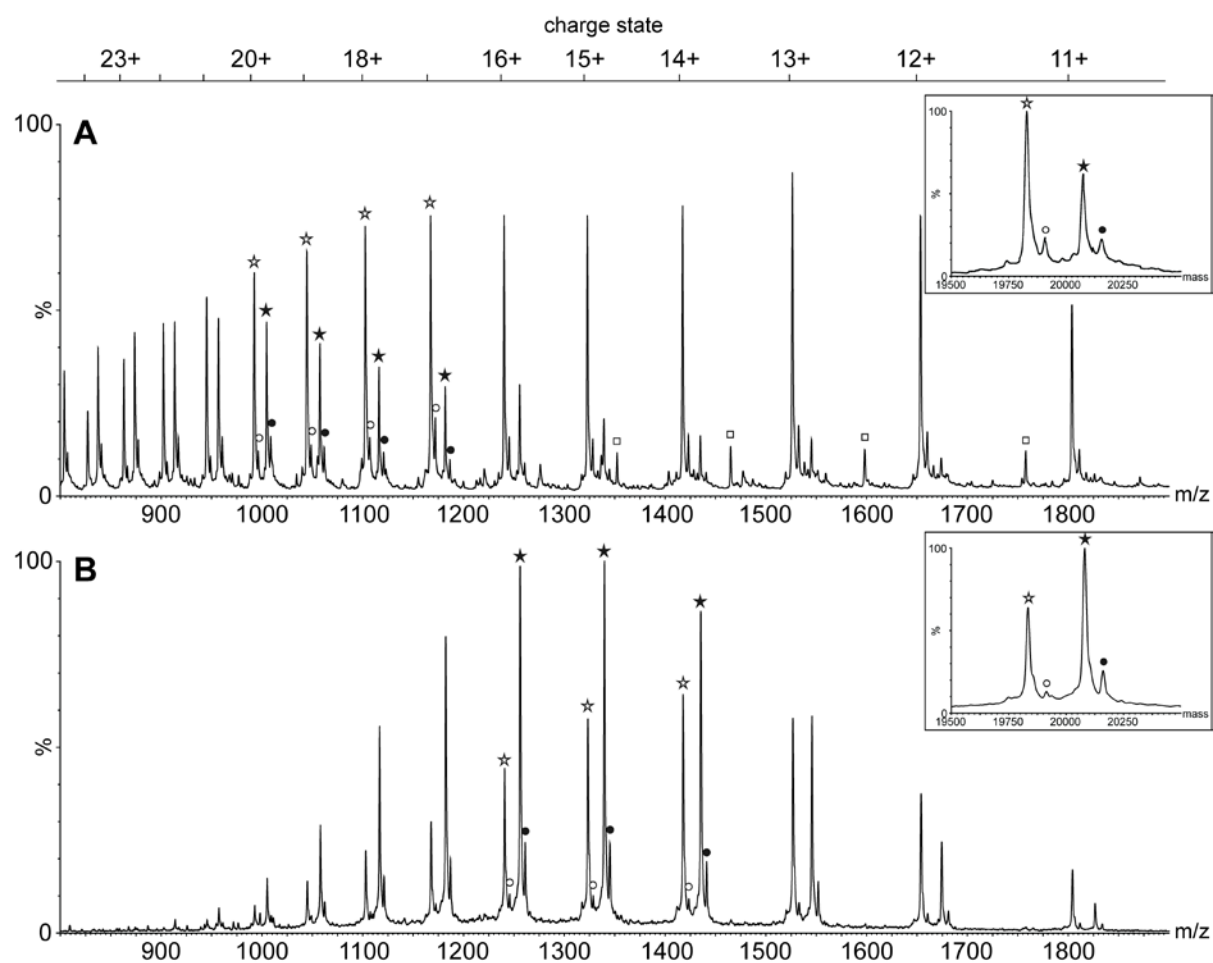


FIGURE 1



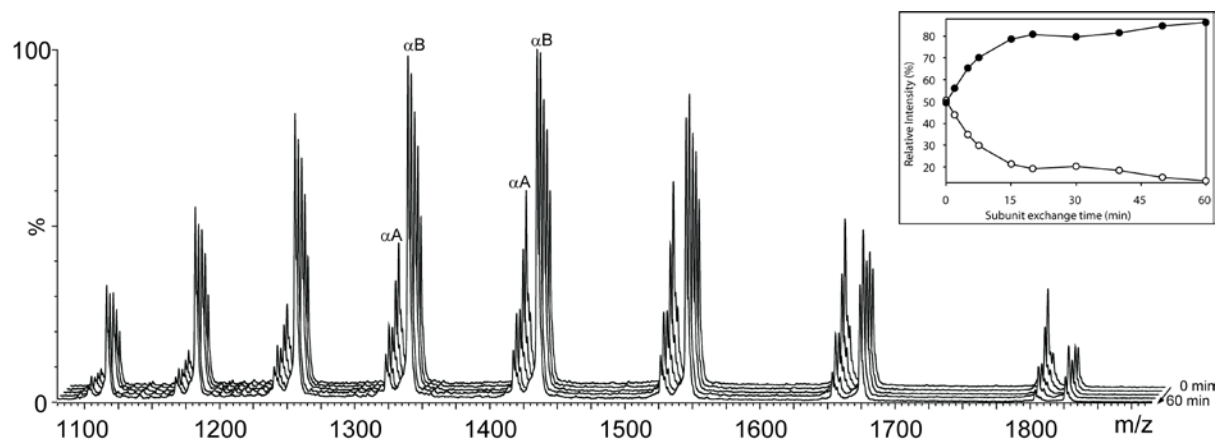


FIGURE 2

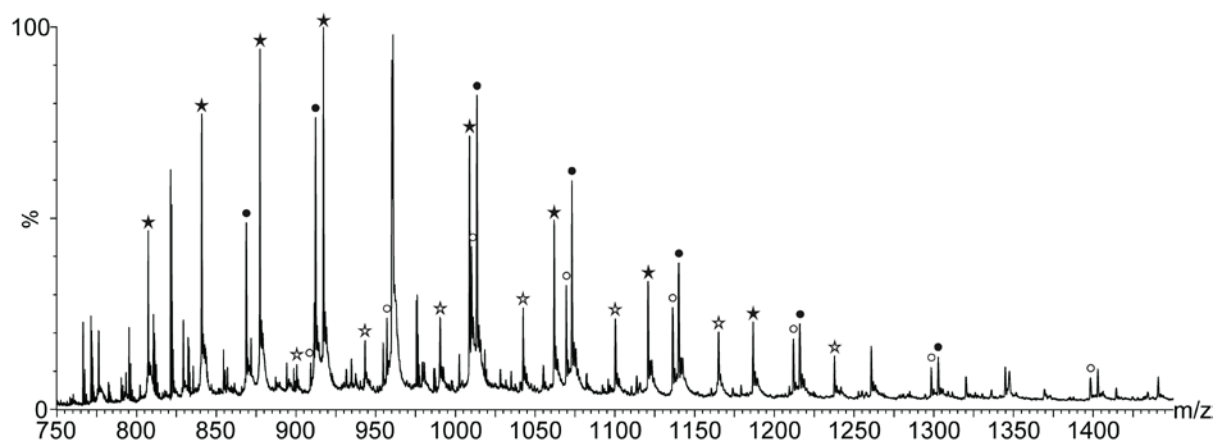


FIGURE 3

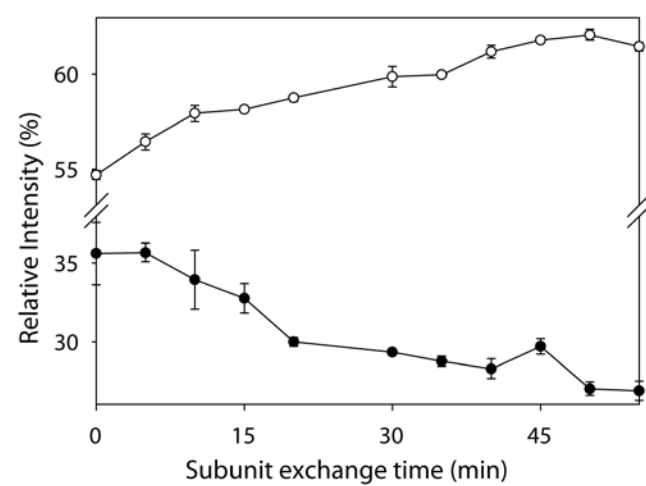


FIGURE 4

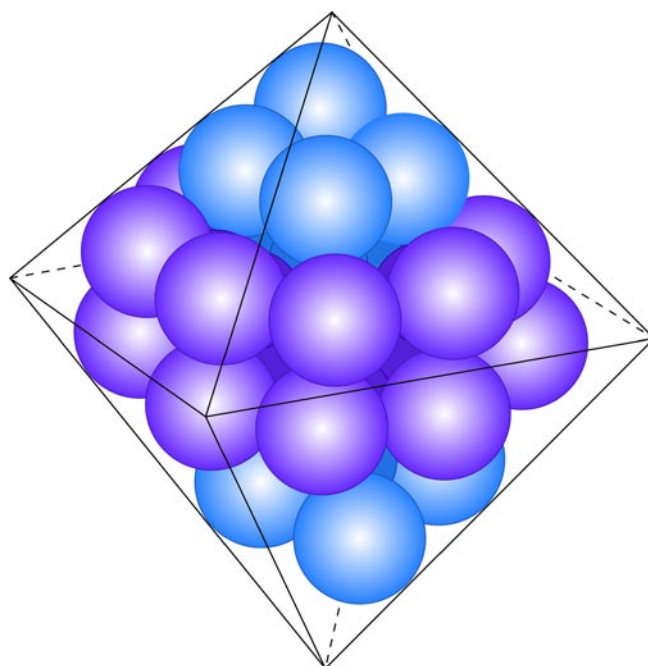


FIGURE 5

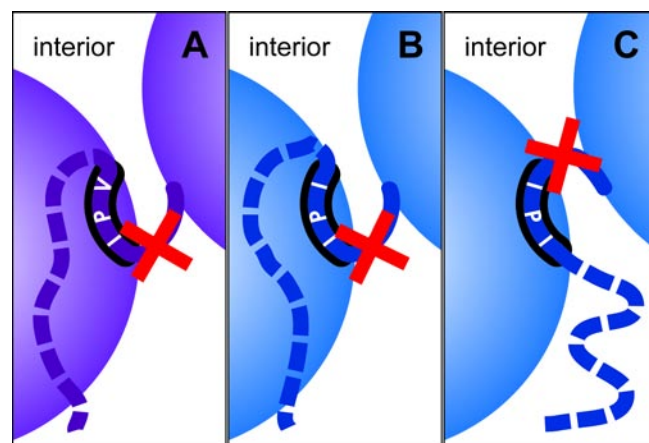


FIGURE 6